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Role of the complement membrane attack complex (C5b-9) in mediating experimental mesangioproliferative glomerulonephritis

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Role of the complement membrane attack complex (C5b-9) in mediating experimental mesangioproliferative glomerulonephritis. Previous studies have demonstrated that most pathologic changes in the anti-thymocyte serum (ATS) model of mesangioproliferative glomerulonephritis are complement-dependent. These include mesangiolysis, glomerular platelet infiltration, mesangial cell proliferation, mesangial cell production of growth factors and phenotypic change to express α-actin, glomerular macrophage infiltrate, mesangial matrix expansion, and proteinuria. The mechanism by which complement mediates these effects has not been defined. Because neutrophils do not participate in the ATS model, we hypothesized that the complement effects observed are consequent to glomerular cell insertion of the C5b-9 membrane attack complex of complement. This hypothesis was tested utilizing PVG rats which exhibit an absence of C6 inherited in an autosomal recessive pattern. C6 deficient (C−) PVG rat serum activated by zymosan produced normal amounts of C5a compared to normocomplementemic (C+) PVG rat controls but no C5b-9. When ATS was induced, C− PVG rats had a significant and marked reduction in mesangiolysis, platelet infiltration, mesangial cell proliferation, α-actin expression, macrophage infiltration, collagen IV deposition, and proteinuria compared to C+ controls. The reduction in each of these parameters was comparable to that achieved by systemic complement depletion of C+ PVG rats with cobra venom factor. These findings establish the role of C5b-9 in mediating each of the complement-dependent features of the ATS model and indicate that C5b-9 accounts for all of the complement-mediated effects observed. This study provides the first documentation of a functional role for C5b-9 in mediating a non-membranous inflammatory type of glomerular injury *in vivo*.

Mesangial cell proliferation is a common cellular response to a variety of different types of glomerular injury in animal models and in humans [1, 2]. Considerable evidence now suggests that proliferation of mesangial cells may be central to the subsequent increase in mesangial matrix and development of glomerulosclerosis [reviewed in 3]. The most extensively studied model of mesangioproliferative glomerulonephritis is the anti-thymocyte serum (ATS) model in rats induced by injection of antibody to the Thy 1.1 antigen expressed on the plasma membrane of mesangial cells [4]. Recent studies in this model have defined the role of growth factors including PDGF and bFGF in mesangial cell proliferation and of TGFβ in the development of glomerulosclerosis [5–9].

Early studies of the ATS model demonstrated that glomerular injury including mesangial cell proliferation and proteinuria were complement-dependent but neutrophil-independent [10]. Subsequent studies have confirmed and extended these observations to document that systemic complement depletion reduces mesangiolysis, mesangial cell proliferation, and glomerular platelet and macrophage influx [11, 12]. Complement depletion also abrogates increases in PDGF, PDGF receptor, bFGF and extracellular matrix in this model [5–7, 13]. However, the mechanism of the complement effect has not been defined.

Nephritogenic consequences of complement activation include generation of chemotactic factors such as C5a and formation of the C5b-9 membrane attack complex [14]. C5a is generally regarded as the principal mediator of lesions involving circulating inflammatory cells, while C5b-9 has been implicated primarily in causing proteinuria without inflammatory cells in models of membranous nephropathy [14]. However, C5b-9 can stimulate production of multiple inflammatory mediators by resident glomerular cells [15–17]. Because several inflammatory cellular processes in the ATS model are complement-dependent and neutrophils are not present, we hypothesized that these events may instead be C5b-9 mediated. Our studies, utilizing a C6 deficient strain of PVG rat, implicate C5b-9 for the first time in mediating inflammatory changes in a model of immune glomerular disease.

Methods

Experimental animals

Three month old male PVG rats weighing 250–300 grams were obtained from two separate vendors. PVG rats with normal complement activity were obtained from Harlan Sprague-Dawley (Cambridge, UK). Age and sex matched complement deficient PVG rats were obtained from Bantin and Kingman Universal (Edmonds, WA). All rats were housed in metabolic cages in an accredited animal facility with free access to normal rat chow and water.

Complement measurements

In order to establish the complement status of PVG rats being studied, the hemolytic activity in serum from each rat was measured by a standard CH_{50} assay [18].

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Individual complement components. In order to determine the activity of the nine individual complement components in PVG serum, tube assays developed by Diamedix Corp. (Miami, FL) were utilized [18, 19]. C3, C5, C6 and C7 were measured using sheep erythrocytes sensitized by the 7s IgG fraction of antibodies to sheep erythrocytes and exposed to predetermined concentrations of guinea pig C1 and human C4 (EAC1gp4hu). Serial dilutions of serum (1:50 to 1:8 $\times$ 10^5 depending on the component being measured) from PVG rats were made in 0.2% gelatin veronal buffer containing 0.3 mM calcium chloride and 2 mM magnesium chloride and 2.5% dextrose (GGVB++) and mixed with 0.1 ml EAC1gp4hu cells (5 $\times$ 10^7 cells/ml) along with 0.05 ml purified human complement components C2 (50 U/ml), C3 (100 U/ml), C5 (50 U/ml), C6 (50 U/ml) and/or C7 (50 U/ml) diluted in GGVB++, omitting the component to be measured. After incubating at 30°C for 30 minutes, in order for C7 to assemble on the erythrocytes, 0.05 ml guinea pig C8 (100 U/ml) and C9 (50 U/ml) in GGVB++ were added. The cells were incubated for an additional 60 minutes at 37°C in order for cell lysis to occur. Unlysed cells were removed by centrifugation and the optical density of the supernatants was measured at 415 nm. Sera obtained from PVG rats with normal CH_{50} levels (C+) were compared with sera from PVG rats with low CH_{50} levels (C−), and results are expressed as a percentage of the hemolysis from C+ serum.

C8 and C9 were measured in much the same way as above. EAC1gp4hu cells (0.1 ml at 5 $\times$ 10^7 cells/ml in GGVB++) were mixed with 0.05 ml of human components C2, C3, C5, C6 and C7 (same concentrations as above) and incubated at 30°C for 30 minutes in order for C1 through C7 to assemble on the erythrocytes. Serial dilutions of PVG serum in GGVB++ (0.05 ml of 5 $\times$ 10^5 to 8 $\times$ 10^6 for C8 and 1 $\times$ 10^4 to 1.6 $\times$ 10^5 for C9) were then added, with either human C8 (100 U/ml) or C9 (50 U/ml), leaving out the component to be measured. The erythrocytes were then incubated at 37°C for an additional 60 minutes in order for lysis to occur. Unlysed cells were removed and the optical density of the supernatants was measured as above.

C1 and C4 were measured using similar assays. C1 activity was measured using sensitized sheep erythrocytes containing human C4 (EAChu4). PVG serum (0.05 ml diluted from 6 $\times$ 10^3 through 9.6 $\times$ 10^4 in GGVB++) and 0.05 ml of EAC4hu cells (5 $\times$ 10^7 cells/ml in GGVB++) were mixed and incubated at 30°C for 20 minutes in order for C1 from the serum to bind. Purified human C2 (0.05 ml at 50 U/ml) was then added to the cells and incubated an additional 10 minutes at 30°C. Guinea pig whole complement (0.05 ml diluted 1:12 in 40 mM EDTA) was then added to the cells along with an additional 0.05 ml of 40 mM EDTA. EDTA was added to bind Ca^{2+} and Mg^{2+} in the GGVB++, which prevents classical pathway components C1, C2 and C4 from binding. Cells were incubated at 37°C for 60 minutes. Unlysed cells were removed by centrifugation, and supernatants were read at 415 nm. C4 activity was measured using the same procedure, except sheep erythrocytes containing guinea pig C1 (EACgp1) were used, and serum was diluted 1:250 through 1:4 $\times$ 10^3.

C2 activity was measured in C+ and C− PVG serum by preparing serial dilutions of serum (1:50 to 1:800) and mixing 0.05 ml with 0.1 ml of EAC1gp4hu cells (5 $\times$ 10^8 cells/ml). Cells were incubated at 30°C for 5 minutes, in order for C2 to bind. Guinea pig whole complement (0.05 ml of a 1:12.5 dilution in 40 mM EDTA) was then added along with 0.05 ml 40 mM EDTA, and cells were incubated an additional 60 minutes at 37°C. Unlysed cells were removed as above, and optical density of the supernatants was measured at 415 nm.

Additional measurements were made of C5 activity using higher concentrations of human C6 (Cal Biochem, San Diego, CA) in the assay, to compensate for the complete deficiency of C6 activity in the C− animals. Results seen in this assay were similar to the results from the C5 assays using the normal concentration of C6.

In measurements of individual complement components, serum from five PVG rats that were complement deficient as judged by CH_{50} titer was compared with a pool of serum obtained from five PVG rats with normal CH_{50} levels. Measurements of C5 levels were also conducted on 30 individual complement deficient animals. In addition to the standard hemolytic assay, hemolytic assays for C5 were also carried out in the presence of up to a fourfold excess of added C6 and also utilizing C5 deficient human serum (Quidel, San Diego, CA) as a C6 source. Results of the complement component assays are expressed as a percentage of the normal PVG pool. C5 levels were also assessed by immunoblotting utilizing a cross-reactive antibody to human C5 (Dako, Roskilde, Denmark). Immunoblots were quantified with a digitized image analysis system.

C6 protein levels were also assessed in PVG rat serum by immunoblotting using a polyclonal antibody to human C6 cross-reactive with rat C6 (gift of Dr. O. Gotze, Gottingen, Germany) as well as a monoclonal antibody to rat C6 (3G11) described elsewhere [20]. In some studies, PVG serum was compared with a pool of fresh normal serum obtained from age, sex and weight matched Sprague-Dawley rats (Simonson Laboratories).

An ELISA assay for rat C6 was also developed utilizing the polyclonal anti-human C6 as the capturing antibody and a biotinylated version of the same antibody as the detecting antibody followed by streptavidin-peroxidase utilizing protocols described elsewhere [20].

C5a generation. C5a generation was measured in duplicate in zymosan activated sera from two C− and two C+ PVG rats using a Boyden chamber assay and human neutrophils as described elsewhere [21]. Serial dilutions from 1:10 to 1:320 were assayed.

C5b-9 complex generation. Finally, to assess the ability of PVG rats to generate C5b-9 complexes 50 μl of C+ PVG, C− PVG or C− PVG serum supplemented with human C6 (50 μg/ml) was further supplemented with 0.5 μg of human C8 labeled with ^{125}I using Bolton-Hunter reagent (1 μg C8 corresponded to approximately 0.7 μCi) and inulin (10 mg/ml). After incubation at 37°C for one hour, inulin was removed by centrifugation (10 min, 100,000 g), and the supernatants were separated by sucrose-density centrifugation (5 to 40% sucrose in phosphate-buffered saline) for 16 hours at 30,000 rpm using a swing-out rotor SW 60 (Beckman Instruments, Munich, Germany). The samples were harvested in 0.5 ml aliquots and radioactivity measured. For control purposes, ^{125}I C8 alone or in the presence of preformed human C5b6 and C7 and C9 (1 μg each) was added to parallel tubes.

Induction of experimental mesangioproliferative glomerulonephritis and experimental design

The ATS model was induced by a single i.v. injection of 1.0 ml/100 g of goat anti-rat Thy 1 antiserum as described in detail

elsewhere [5–9]. These groups of animals were studied: Group A ($N = 6$) were normocomplementemic PVG (C+ PVG) rats given ATS; Group B ($N = 6$) were complement deficient PVG rats (C− PVG) given ATS; and Group C ($N = 6$) were normocomplementemic PVG rats given ATS and systemically complement depleted (C+ PVG/CVF) by administration of cobra venom factor. Urine protein excretion was measured from zero to 24 hours in metabolic cages with free access to food and water. Biopsies were performed under ether anesthesia 24 hours and five days after disease induction and examined by light, immunofluorescence, and electron microscopy as described below. CH_{50} measurements were performed before disease induction and again at day five.

Complement depletion

C+ PVG rats in group C were depleted of C3 with cobra venom factor (CVF; naja naja kauthia, Diamedix). Rats were injected with 30 U i.p. every eight hours on the day prior to disease induction and then with 30 U twice daily on day zero and one, 50 U twice daily on day two, 70 U twice daily on day three, and 90 U twice daily on day four prior to sacrifice on day five.

Renal morphology

Light microscopy. Tissue for light microscopy and immunoperoxidase staining was fixed in methyl Carnoy's solution and embedded in paraffin. Four micron sections were stained with periodic acid-Schiff (PAS) and counterstained with hematoxylin.

Immunocytochemistry. Four micron sections of methyl Carnoys fixed biopsy tissue were stained by an indirect immunoperoxidase technique. Antibodies used included:

19A2 (Coulter, Hialeah, FL), a murine monoclonal IgM antibody against human PCNA, which is expressed by actively proliferating cells [22]

ED-1 (Bioproducts for Science, Indianapolis, IN), a murine monoclonal IgG to a cytoplasmic antigen present in rat monocytes, macrophages, and dendritic cells [23]

RP-3 (gift of F. Sendo, Yamagato, Japan) a murine monoclonal IgG antibody to rat neutrophils [24]

PL-1, a murine monoclonal antibody against rat platelets (gift of W.W. Bakker, Groningen, The Netherlands) [25]

A murine monoclonal antibody to an NH2-terminal synthetic decapeptide of alpha-smooth muscle actin (gift of G. Gabbiani, Geneva, Switzerland) [26]

A biotinylated IgG fraction of polyclonal goat anti-mouse type IV collagen (Southern Biotech, Birmingham, AL)

Macrophages, proliferating cells, and neutrophils are reported as the mean number of cells per glomerulus positively stained by the respective antibodies as reported elsewhere [5, 11, 13]. Platelet and alpha-smooth muscle actin staining was quantitated by counting the mean number of quadrants of the glomerulus which stained positively, as reported elsewhere [27].

Immunofluorescence. Six micron sections of frozen tissue were stained with FITC conjugated antibodies to goat IgG (Cappel, Organan Teknika Corp., Durham, NC), human C3d (Dako Corp., Carperteria, CA) [28], and a biotinylated anti-rat C5b-9 complexed to a FITC-bound streptavidin peroxidase system [20, 29]. Glomeruli were graded 0 to 4 based on the intensity of staining.

To further assess the possible effects of reduced levels of C2 in C− PVG rats, deposition of C3d was quantitated by confocal laser microscopy. Four micromolar sections of frozen kidney were stained for rat C3d as described above. Sections were then scanned by confocal microscopy using an ACSA Ultima Interactive Laser Cytometer (Meridian Instruments, Okemos, Michigan, USA), which permits quantitative analysis of fluorescence intensity. An image field of 3.2 mm^2 was scanned for each section; where less than 15 glomeruli were present two fields were scanned. Using the Meridian image analysis system, the fluorescence intensity over individual glomeruli was then analyzed. Data from a minimum of 13 individual glomeruli per animal were averaged. Results are expressed as the mean ± standard error of average fluorescence units emitted per glomerulus.

Electron microscopy. Portions of biopsy specimens were fixed in 1/2 strength Karnovsky's solution and processed as previously described [27]. Thin sections were mounted on coated nickel grids and stained with osmium followed by uranyl acetate. Sections were examined using a Phillips 410 electron microscope.

Other variables measured. Urine was collected in all animals on the day following induction of disease and protein content was measured by a sulfosalicylic acid method [30]. Serum collected prior to disease induction and at the time of sacrifice was assayed for CH_{50} levels.

Statistical analysis. Results are presented as the mean ± standard error unless otherwise noted. Comparisons between C+ PVG and C− PVG or C+ PVG/CVF animals were done by ANOVA and two-tailed unpaired Student's *t*-test or Mann Whitney test, as appropriate [31]. Statistical significance was defined as $P < 0.05$.

Table 1. Levels of individual rat complement components in pooled sera from five C− PVG rats expressed as a percentage of levels in a pool of five age and sex matched C+ PVG controls

Complement measurement	Present study	Leenaerts et al [31]
CH_{50}	<1%	<1%
AP50	ND	2.2
C1q	ND	88
C1	130	70
C4	60	81
C2	15	ND
C3	80	95
C5[a]	20 ± 13.5	88
C6	<1	<1
C7	148	70
C8	106	ND
C9	96	ND
D	ND	96
B	ND	100
H	ND	105

C5 assays were done on 30 individual C− PVG rats compared to a pool of C+ PVG rat serum. Abbreviation ND is not detectable.

[a] Data are mean ± SD

Results

Complement measurements

The values for individual complement components in C− PVG rats as a percentage of levels in C+ PVG rats are given in Table 1. C− PVG rats had no detectable C6 by functional assay. C6 was also undetectable in C− PVG rats by both immunoblotting and ELISA assays utilizing a monoclonal antibody specific for rat C6. All other individual complement components tested were within

Table 2. Summary of results on day one or five in C+ PVG rats, C− PVG rats, and C+ PVG rats complement-depleted with CVF

Variables measured	Day	C+ PVG	C− PVG	C+ PVG/CVF
CH_{50} % *C+ PVG*	1	100	<1%	<1%
	5	100	<1%	14 ± 8.7
Urine protein *mg/day*	1	32 ± 15	15 ± 6[a]	19 ± 7[a]
Glomerular cellularity *cells/glom*	1	52 ± 5.6	51 ± 4.2	53 ± 1.8
Cell proliferation *PCNA + cells/glom*	5	3.0 ± 0.4	0.53 ± 3[a]	0.21 ± 0.1[a]
Alpha-actin (0–4+)	5	2.6 ± 0.2	0.23 ± 0.1	0.9 ± 0.5[a]
Platelets *cells/glom*	1	8.8 ± 2.0	1.7 ± 0.6	1.6 ± 0.5[a]
Macrophages *ED1 cells/glom*	1	6.0 ± 0.6	1.1 ± 0.2	0.88 ± 0.04[a]
Neutrophils *RP3 cells/glom*	1	0.0	0.0	0.0
	5	0.0	0.0	0.0
Collagen IV (1–4+)	5	2.6 ± 0.63	1.4 ± 0.69[a]	0.17 ± 0.62[a]
Immunofluorescence				
Goat IgG (0–4+)	1	4+	4+	4+
	5	2+	3+	3+
C3d (0–4+)	5	3+	3+	0.5
C5b-9 (0–4+)	5	2+	0	0

Values are mean ± SE.
[a] $P < 0.05$ vs. C+ PVG

Table 3. Results of measurements of C5a generation in a Boyden chamber assay expressed as chemotaxis index and leading front at different dilutions of C+ and C− PVG serum

Group/dilution	Chemotaxis index	Leading front *μm*
C+ PVG		
1:10	67	170
1:20	68	160
1:40	56	160
1:80	25	88
1:160	22	70
1:320	20	68
C− PVG		
1:10	72	160
1:20	66	185
1:40	58	160
1:80	23	88
1:160	27	92
1:320	24	91
Human serum (1:20)	42	130
Medium alone	20	63

Two samples were studied in duplicate for each group. No significant differences between C+ and C− PVG sera were detected at any dilution. Values are means of duplicate samples.

the normal range (>50% of a normal pool) except for C5 (20%) and C2 (15%) (Table 1). CH_{50} levels in rats treated with cobra venom factor were less than 1% of normal on day one and less than 15% of normal on day five (Table 2).

To further assess the significance of the reduced C2 levels in mediating the ATS lesion, rat C3d deposits in glomeruli were quantitated by confocal laser microscopy at day five. The results demonstrated no significant reduction in glomerular C3d deposition in C− PVG rats compared to controls (mean 1410 ± 296 SD fluorescent units, $N = 7$ vs. 1742 ± 259 units, $N = 8$; $P > 0.05$) suggesting equivalent C3 activation in both groups. CVF treatment did significantly reduce glomerular C3d deposits (767 ± 40, $P < 0.01$).

To further assess the apparent deficiency in C5 by standard hemolytic assay, hemolytic assays were repeated with increased quantities of human C6 added (up to a fourfold excess) and with C5 deficient human serum as a C6 source with similar results. Both assays gave C5 values of about 20% of a C+ PVG pool. However, immunoblotting of C5 using a cross-reactive antibody to human C5 demonstrated similar or greater quantities of C5 protein in C− PVG rats compared to C+ controls (data not shown). When C5a generation was measured in C+ and C− PVG serum, no significant difference between C− and C+ serum in chemotaxis index or leading front could be demonstrated at any dilution tested (Table 3).

Finally, the ability of C− PVG serum to generate C5b-9 complexes following vigorous alternative complement pathway activation with inulin was assessed by measuring incorporation of ^{125}I-labeled C8 into C5b-9. In these studies, C6 was repleated to a level of 50 μg/ml utilizing purified human C6. In C+ PVG sera, 19% of the radiolabeled C8 appeared in the 19S fraction corresponding to the sedimentation of C5b-9 formed from preformed C5b-6 with addition of purified C7, ^{125}I C8 and C9. The remaining labeled C8 was found in a 9S fraction corresponding to the position of the ^{125}I C8 alone. In C− PVG sera no radioactivity was seen in the 19S region, and all radioactivity appeared in the 9S fraction. In C− PVG serum supplemented with human C6, 14% of the radioactivity (74% of that in the C+ serum) was found in the 19S fraction and the remainder in 9S. These data represent the mean of two independent experiments.

Glomerular morphology

The results of light and immunofluorescence studies are summarized in Table 2. By light microscopy there were no significant differences in overall appearance between the three groups. Intrinsic glomerular cell counts were not different between the groups (Table 2). However, mesangiolysis was apparent in most glomeruli by light and electron microscopy in C+ PVG rats but was not detected in C− PVG rats or CVF treated rats (see below).

Glomerular cell proliferation was not present at day one but was demonstrable at day five in C+ PVG animals. However, proliferation was reduced by over 90% in C− and CVF treated rats, confirming earlier studies which demonstrated that proliferation in this model is complement-dependent [11, 12]. Glomerular cell proliferation was accompanied by a phenotypic change with *de novo* expression of α-smooth muscle actin in C+ PVG rats as reported previously in other strains [32]. No significant α-actin expression was seen in C− or CVF treated rats (Table 2).

Platelet infiltrates were also seen at day one in C+ PVG rats but were markedly and equally reduced in C− and CVF treated groups (Table 2, Fig. 1), also confirming the findings of previous studies [11, 12].

Similarly, a prominent macrophage infiltrate was seen as early as day one in C+ PVG rats but was markedly reduced in C− PVG rats and was essentially abolished by CVF treatment (Table 2).

Finally, type IV collagen staining as an index of mesangial

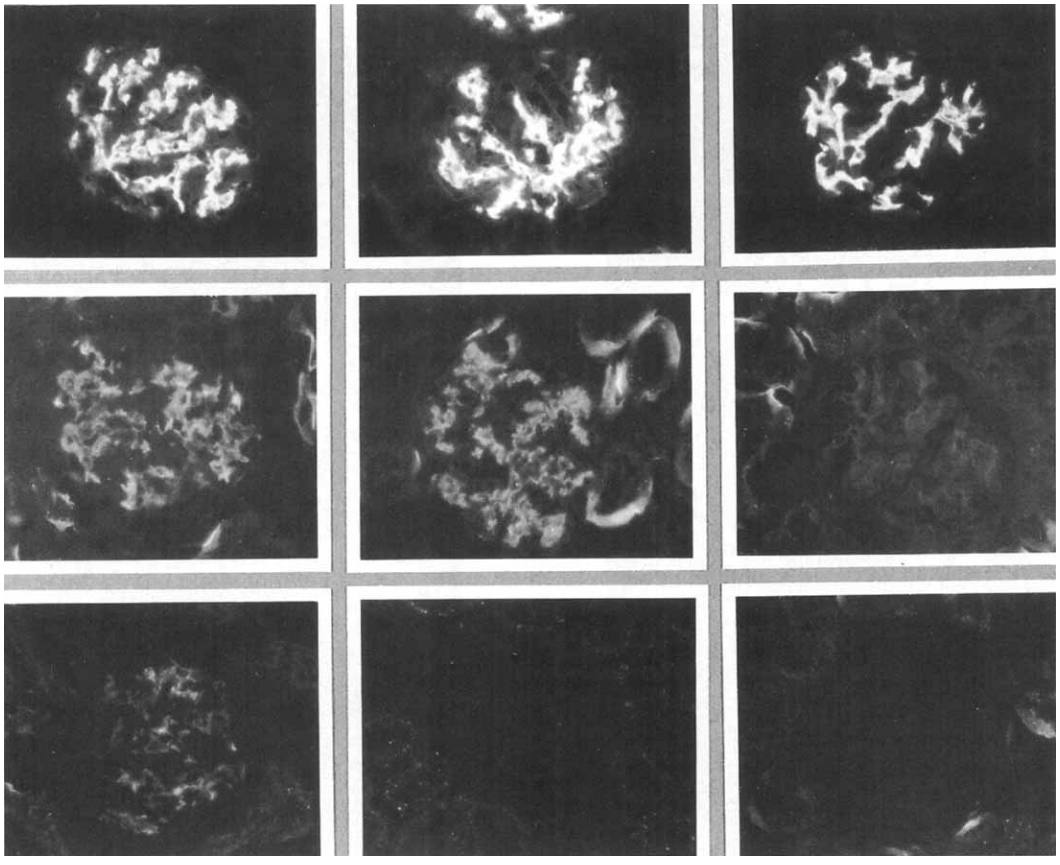

Fig. 1. *Immunofluorescence photomicrographs of representative glomeruli at day 5 stained for goat IgG (top row), rat C3 (middle row) and rat C5b-9 (bottom row).* C+ PVG rats (left column) show 4+ staining for goat IgG, 3+ C3 and 2+ C5b-9. C− PVG rats (middle column) show staining for IgG and C3 similar to that in the C+ rats but no staining for C5b-9. CVF treated C+ rats (right column) show normal deposits of goat IgG but no staining for C3 or C5b-9. Original magnifications 400×.

matrix expansion was increased at day five in C+ PVG rats, as previously reported in other strains [13]. Collagen IV staining was substantially reduced in C− PVG rats and also by CVF treatment (Table 2).

Immunofluorescence staining revealed prominent and equal deposition of goat IgG in all three groups at day one (Table 2, Fig. 1). Rat C3d staining was equivalent in C+ and C− PVG rats but was reduced by CVF treatment (Table 2). Quantitation of C3d IF by confocal laser microscopy confirmed no significant reduction in C3d staining in C− PVG rats compared to C+ controls (see above). Rat C5b-9 staining was prominent at day five in C+ PVG rats and was in a predominantly mesangial distribution (Table 2, Figure 1). C5b-9 staining was absent in C− or CVF treated groups (Table 2, Figure 1).

Ultrastructural studies

Electron microscopic studies of glomeruli from representative animals in each group revealed changes similar to those described in previous studies of the ATS model in other strains [4, 5]. In contrast to the normal rat glomerulus, there were prominent platelet infiltrates at day one in the C+ PVG animals (Fig. 2) as well as a substantial amount of mesangiolysis (Fig. 3). Platelet infiltration was markedly diminished on day one in C− PVG rats, which exhibited no detectable mesangiolytic changes by electron microscopy (Fig. 4).

Urine protein excretion

Proteinuria occurred on day one in C+ PVG rats (32 ± 10 mg/day) and was substantially reduced in both C− PVG rats and CVF treated animals (Table 2).

Discussion

Our findings demonstrate a marked reduction in severity of all structural and functional consequences of glomerular injury in the ATS model induced in C− PVG rats compared to C+ PVG controls. The amelioration of disease in complement deficient animals was comparable to that achieved by generalized complement depletion in C+ PVG rats induced by CVF and confirms previous studies of the role of complement in this model [11, 12]. We believe the difference in disease manifestations between C+ and C− PVG rats is primarily due to the inability of C− rats to form C5b-9 membrane attack complexes because of the total absence of C6 in these animals. However, this contention requires that the only significant difference between these groups be in the ability to form C5b-9.

Leenaerts et al were the first to describe C6 deficiency in the

Fig. 2. *Electron micrograph of a C+ PVG rat glomerulus showing a focally prominent influx of platelets one day after administration of ATS antiserum. Magnification 11,000×.*

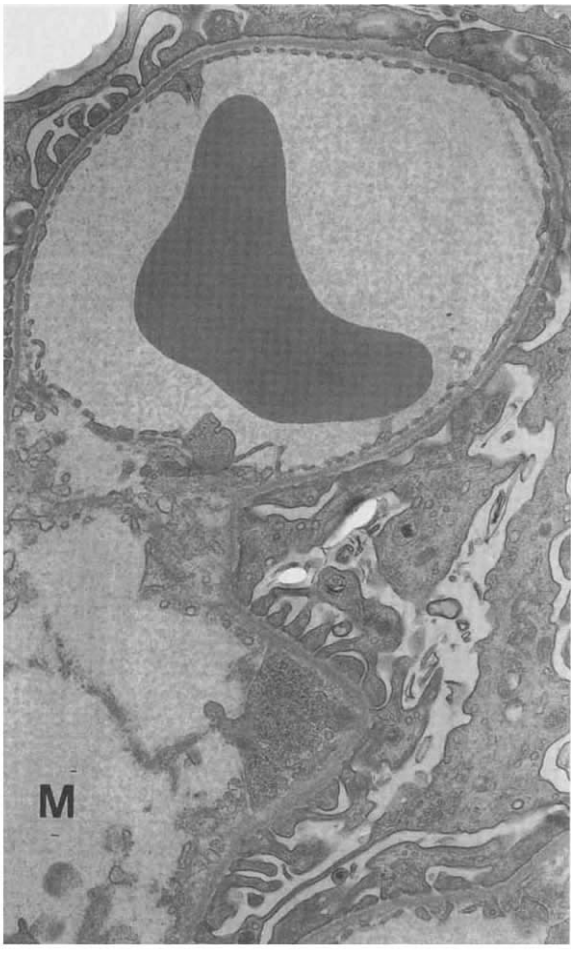

Fig. 3. *Electron micrograph of a C+ PVG rat glomerulus one day after administration of ATS.* There is marked mesangiolysis (M) with preservation of glomerular endothelium and capillary wall structures. Magnification 10,000×.

PVG rat utilizing animals obtained from the same source as the C− PVG rats utilized in this study [33]. Their studies document a total lack of C6 inherited as an autosomal recessive trait. In addition, Leenaerts et al report full reconstitution of CH_{50} activity with purified human C6 and normal levels of individual rat complement components C1–C7 (excluding C2), as well as normal levels of factors D, B and H [33]. Others have confirmed a deficiency or absence of C6 in other PVG strains [34, 35]. Finally, Leenaerts et al reported these animals to be phenotypically and immunologically normal with full acceptance of skin grafts between C− PVG and C+ PVG animals [33].

Our results confirm the total absence of C6 in C− PVG rats by hemolytic assay, immunoblotting, and ELISA assays. We have also demonstrated the inability of C− PVG rats to make detectable C5b-9 complexes following vigorous complement activation *in vitro* or following antibody induced glomerular injury *in vivo*. C5b-9 formation by C+ PVG rat controls *in vivo* was comparable to that seen in other rat strains with normal CH_{50} levels. We also found that individual complement components other than C6 were greater than 50% of values in C+ PVG animals except for C2 and C5, which were 15 and 20% of normal values, respectively. With regard to a possible contribution of low C2 levels to our results, the presence of equal amounts of C3d deposition in glomeruli of C− and C+ PVG rats quantitated by confocal laser microscopy in this and other studies of experimental membranous nephropathy in C− PVG and C+ PVG rats (data not shown) suggests that C3 activation in glomeruli was not limited by C2. This conclusion would apply regardless of whether C3 activation occurs via the classical pathway of complement activation where reduced C2 might be limiting, or via the alternative pathway as may occur with damaged cells in other settings [36, 37].

A larger concern would be that reduced C5 levels led to impaired generation of C5a in the C− PVG group. However, measurements of C5a generation revealed no significant differences between C− and C+ PVG sera, and no neutrophils were seen in either group confirming previous findings by ourselves and others that neutrophils do not participate in the ATS lesion [4, 10, 11]. Moreover, the observation that C− PVG serum supplemented with human C6 generated 74% of the C5b-9 complexes generated by C+ PVG serum suggests that C5b-9 generation in

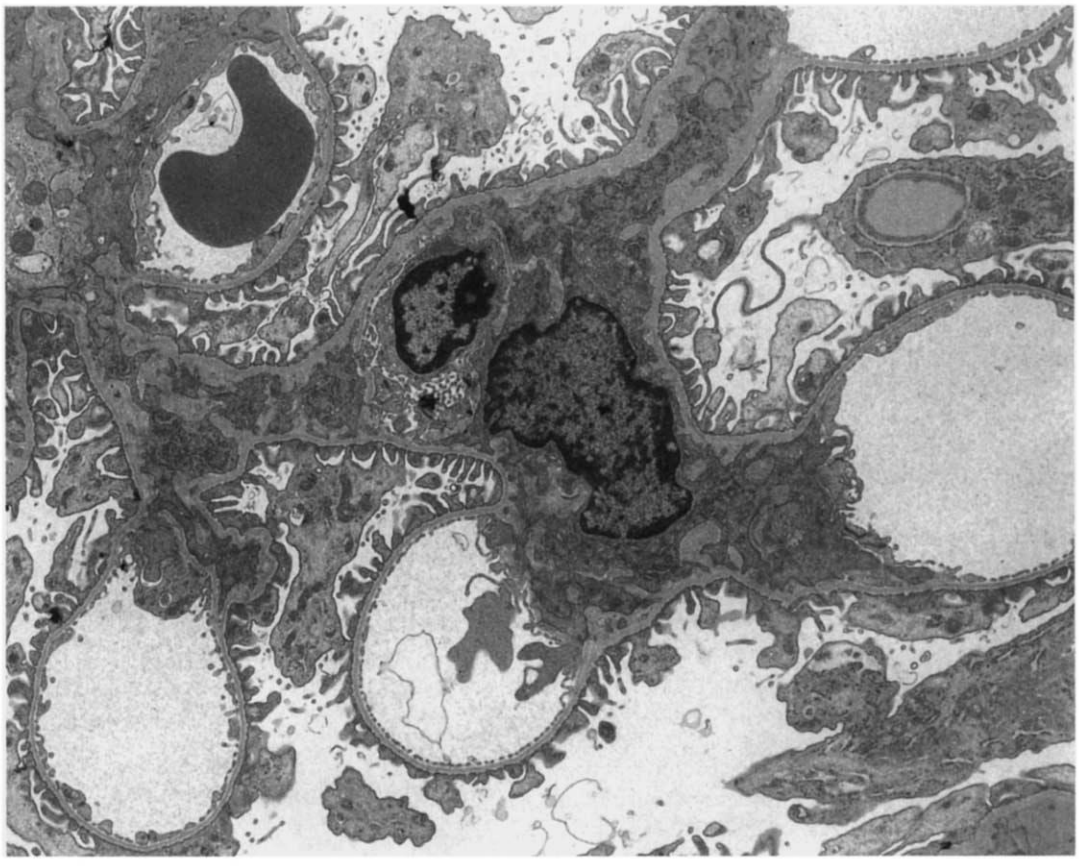

Fig. 4. *Electron micrograph of a C− PVG rat glomerulus one day after administration of ATS antiserum.* Mesangiolysis and platelet infiltrates are absent. Magnification 5600×.

the presence of C6 is normal in C− PVG rats. The fact that C5b-9 generation was not 100% of levels in C+ PVG rats probably reflects the less efficient utilization of human C6 in generating C5b-9 in rat serum compared to C+ PVG rats where all rat distal complement components are present. Thus, we believe the ability of C− PVG rats to generate normal amounts of C5a and near normal amounts of C5b-9 when human C6 is provided suggests that the principal difference between C− and C+ PVG rats with regard to nephritogenesis lies in the absence of C6 in C− rats and consequently the inability of these animals to generate C5b-9.

Previous studies utilizing generalized complement depletion induced with CVF or administration of a soluble CR1 molecule have documented that several features of the ATS model are dependent on complement activation induced by the binding of IgG antibody to the mesangial cell membrane [11, 12]. These features include mesangiolysis, glomerular localization of platelets and macrophages, mesangial cell proliferation and consequent phenotypic changes including expression of α-smooth muscle actin, PDGF, PDGF receptors and deposition of type IV collagen [11–13]. Each of these features studied (PDGF and PDGF receptors were not studied) was also markedly reduced in C− PVG rats compared to C+ controls, and in most cases the differences observed were comparable to those induced by administration of CVF, suggesting that C5b-9 was not simply contributing to these phenomena but in fact accounts for most of the complement-dependent features observed.

With regard to the mechanism of the C5b-9 effect in the ATS model, several processes are probably involved. In lytic amounts, C5b-9 can kill nucleated glomerular cells, a process which likely accounts for the mesangial lysis in C+ PVG animals [15, 38]. Cell lysis may also promote release of intracellular cytokines and growth factors, such as bFGF, which has been shown to play a role in early mesangial cell proliferation in the ATS model [6, 7]. However, nucleated cells are generally resistant to lysis by C5b-9. Rather, they may exhibit features of cell activation in response to sublytic C5b-9, leading to overproduction of several inflammatory mediators that likely contribute to glomerular disease [15–17, 38]. Thus, sublytic C5b-9 can induce mesangial cells *in vitro* to increase production of prostaglandins, oxidants, growth factors such IL-1 and IL-8, cytokines such as TNF and extracellular matrix components (collagen IV, fibronectin) [15–17, 39, 40]. It is therefore likely that C5b-9 attack on mesangial cells *in vivo* induced by deposition of anti-Thy 1 antibody normally elicits most or all of these responses which were greatly diminished or absent in C− PVG rats.

In terms of the other specific effects observed in this study, platelet localization is likely mediated by complement through C3b (CR1) receptors present on rat (but not human) platelets, and platelets may aggregate in response to local stimuli such as collagen exposure in areas of antibody/complement mediated tissue injury [41–45]. Documentation of a role of C5b-9 in this process suggests that tissue injury may be more important than

C3b receptors in determining platelet localization *in vivo*. Because mesangial cell proliferation is related to platelets, the proliferative phase of this lesion is generally a complement-dependent phenomenon [11, 12]. In our study, cell proliferation was reduced by 80% in C− PVG rats and by over 90% in CVF treated C+ PVG animals, suggesting that most of the complement effect on cell proliferation is C5b-9 mediated. In addition to the platelet related effects, C5b-9 may cause proliferation through a direct effect [46], through mediating release of growth factors from lysed cells [6, 7], or by stimulating release of growth factors such as IL-1 directly through sublytic effects [16].

Glomerular macrophage infiltration was also reduced in C− PVG rats. Glomerular macrophage recruitment is also multifactorial. Complement-derived chemotactic factors may recruit monocyte/macrophages, macrophages exhibit C3b receptors, and sublytic complement attack on glomerular cells may induce release of other molecules directly chemotactic for monocytes such as IL-1, TNF and monocyte chemotactic peptide [reviewed in 39]. Moreover, complement mediated injury may lead to up-regulation of leukocyte adherence molecules for monocytes and their ligands such as ICAM-1 and LFA-1 [47]. Monocytes are not only involved in mediating acute glomerular injury [48] but are also potent sources of TGFβ, which has been established to mediate matrix expansion and glomerulosclerosis in this model [8, 9]. It is noteworthy that in C− PVG rats not only were a host of structural lesions characteristic of the anti-Thy 1 model modified, but also proteinuria was reduced by over 50%, demonstrating that the cellular events described must also have important functional consequences that were also dependent on C5b-9.

In summary, despite ample evidence for effects of sublytic C5b-9 in mediating injury and activation of glomerular cells *in vitro*, our study provides the first direct evidence that C5b-9 mediates several manifestations of glomerular injury in an inflammatory model of glomerulonephritis *in vivo*. C5b-9 dependent phenomena included mesangiolysis, platelet localization, cell proliferation, cell phenotype change, macrophage infiltration, matrix expansion and proteinuria. These observations extend previous studies which have documented a pathogenic role for C5b-9 only in mediating changes in urinary protein excretion in non-inflammatory models of membranous nephropathy [49]. They also provide several potential mechanisms for the postulated nephritogenic potential of C5b-9 in human glomerular diseases associated with mesangial cell proliferation [50, 51].

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References

1. JOHNSON RJ: The glomerular response to injury: Progression or resolution? (Nephrology Forum) *Kidney Int* 45:1769–1782, 1984
2. ALPERS CE, HUDKINS KL, GOWN AM, JOHNSON RJ: Enhanced expression of "muscle-specific" actin in glomerulonephritis. *Kidney Int* 41:1134–1144, 1992
3. ENG E, FLOEGE J, YOUNG BA, ALPERS CE, COUSER WG, JOHNSON RJ: Is mesangial cell proliferation required for extracellular matrix expansion in glomerular disease? *Contrib Nephrol* 107:156–162, 1994
4. YAMAMOTO T, WILSON CB: Quantitative and qualitative studies of antibody-induced mesangial cell damage in the rat. *J Immunol* 32: 514–525, 1987
5. IIDA H, SEIFERT R, ALPERS CE, GROWNWALD RGK, PHILLIPS PE, PRITZL P, GORDON K, GOWN AM, ROSS R, BOWEN-POPE DF, JOHNSON RJ: Platelet-derived growth factor (PDGF) and PDGF receptor are induced in mesangial proliferative nephritis in the rat. *Proc Natl Acad Sci USA* 88:6560–6564, 1991
6. FLOEGE J, ENG E, LINDER V, ALPERS CE, YOUNG BA, REIDY MA, JOHNSON RJ: Rat glomerular mesangial cells synthesize basic FGF: Release, upregulated synthesis and mitogenicity in mesangial proliferative glomerulonephritis. *J Clin Invest* 90:2362–2369, 1992
7. FLOEGE J, ENG E, YOUNG BA, ALPERS CE, BARRETT TB, BOWEN-POPE DF, JOHNSON RJ: Infusion of PDGF or basic FGF induces selective glomerular mesangial cell proliferation and matrix accumulation in rats. *J Clin Invest* 92:2952–2962, 1993
8. OKUDA S, LANGUINO LR, RUOSLAHTI E, BORDER WA: Elevated expression of transforming growth factor-β and proteoglycan production in experimental glomerulonephritis. *J Clin Invest* 86:453–462, 1990
9. BORDER WA, NOBLE NA, YAMAMOTO T, HARPER JR, YAMAGUCHI Y, PIERSCHBACHER MD, RUOSLAHTI E: Natural inhibitor of transforming growth factor-β protects against scarring in experimental kidney disease. *Nature* 360:361–364, 1992
10. YAMAMOTO T, WILSON CB: Complement dependence of antibody-induced mesangial cell injury in the rat. *J Immunol* 138:3758–3765, 1987
11. JOHNSON RJ, PRITZL P, IIDA H, ALPERS CE: Platelet-complement interactions in mesangial proliferative nephritis in the rat. *Am J Pathol* 138:313–321, 1990
12. COUSER WG, JOHNSON RJ, YOUNG BA, YEH CG, TOTH CA, RUDOLPH AR: The effects of soluble recombinant CR1 on complement-mediated experimental glomerulonephritis. *J Am Soc Nephrol* 5:1888–1894, 1995
13. FLOEGE J, JOHNSON RJ, GORDON K, IIDA H, PRITZL P, YOSHIMURA A, CAMPBELL C, ALPERS CE, COUSER WG: Increased synthesis of extracellular matrix in mesangial proliferative nephritis. *Kidney Int* 40:477–488, 1991
14. COUSER WG: The pathogenesis of glomerulonephritis. *Kidney Int* 44(Suppl 42):S19–S26, 1993
15. ADLER S, BAKER PJ, JOHNSON RJ, OCHI RF, PRITZL P, COUSER WG: Complement membrane attack complex stimulates production of oxygen metabolites by cultured rat mesangial cells. *J Clin Invest* 77:762–777, 1986
16. LOVETT DH, HÄNSCH G-M, GOPPELT M, RESCH K, GEMSA D: Activation of glomerular mesangial cells by the terminal membrane attack complex of complement. *J Immunol* 138:2473–2480, 1987
17. SCHONERMARK M, DEPPISCH R, RIEDASCH G, ROTHER K, HÄNSCH GM: Induction of mediator release from human glomerular mesangial cells by the terminal complement components C5b-9. *Int Arch Allergy Appl Immunol* 96:331–337, 1991
18. NEILSON RA, JENSEN J, GIGLI I, TAMURA N: Methods for the separation, purification, and measurement of nine components of hemolytic complement in guinea pig serum. *Immunochemistry* 3:111–135, 1966
19. VROON DH, SCHULTZ DR, ZARCO RM: The separation of nine components and two inactivators of components in human serum. *Immunochemistry* 7:43–61, 1970
20. SCHULZE M, BAKER PJ, PERKINSON DT, JOHNSON RJ, OCHI RF, STAHL RAK, COUSER WG: Increased urinary excretion of C5b-9 distinguishes passive Heymann nephritis in the rat. *Kidney Int* 35:60–68, 1989
21. BRENNEIS H, SCHMIDT A, BLAAS-MAUTNER P, WÖRNER I, LUDWIG R, HÄNSCH GM: Chemotaxis of polymorphonuclear neutrophils (PMN)

in patients suffering from recurrent infection. *Eur J Clin Invest* 23:693–698, 1993
22. KURKI P, VANDERLAAN M, DOLBEARE F, GRAY J, TAN EM: Expression of proliferating cell nuclear antigen (PCNA)/cyclin during the cell cycle. *Exp Cell Res* 166:209–219, 1986
23. DIJKSTRA CD, DOPP EA, JOLING P, KRAAL G: The heterogeneity of mononuclear phagocytes in lymphoid organs: Distinct macrophage subpopulations in the rat recognized by monoclonal antibodies ED1, ED2 and ED3. *Immunology* 54(3):589–599, 1985
24. SEKIYA S, GOTOH S, YAMASHITA T, WATANABE T, SAITOH S, SENDO F: Selective depletion of rat neutrophils by *in vivo* administration of a monoclonal antibody. *J Leukocyte Biol* 46:96–102, 1989
25. BAGCHUS WM, JEUNINK MF, ROZING J, ELEMA JD: A monoclonal antibody against rat platelets. I. Tissue distribution *in vitro* and *in vivo*. *Clin Exp Immunol* 75:317–323, 1989
26. SKALLI O, ROPRAZ P, TRZECIAK A, BENZONANA G, GILLESSEN D, GABBIANI G: A monoclonal antibody against α-smooth muscle actin: A new probe for smooth muscle differentiation. *J Cell Biol* 103:2787–2796, 1986
27. YOUNG BA, JOHNSON RJ, ALPERS C, ENG E, GORDON K, FLOEGE J, COUSER WG: Cellular events in the evolution of experimental diabetic nephropathy. *Kidney Int* 47:935–944, 1995
28. SCHULZE M, PRUCHNO CJ, BURNS M, BAKER PJ, JOHNSON RJ, COUSER WG: Glomerular localization of C3c indicates on-going immune deposit formation and complement activation in experimental glomerulonephritis. *Am J Pathol* 142:179–187, 1993
29. KERJASCHKI D, SCHULZE M, BINDER S, KAIN R, OJHA PP, SUSANI M, HORVAT R, BAKER PJ, COUSER WG: Transcellular transport and membrane insertion of the C5b-9 membrane attack complex of complement by glomerular epithelial cells in experimental membranous nephropathy. *J Immunol* 143:546–552, 1989
30. BRADLEY GM, BENSON ES: Examination of the urine, in *Todd-Sanford Clinical Diagnosis by Laboratory Methods*, edited by DAVIDSON I, HENRY JB, (15th ed), WB Saunders, Philadelphia, 1974, pp 74–75
31. WALLENSTEIN S, ZUCKER CL, FLEISS JL: Some statistical methods useful in circulation research. *Circ Res* 47:1–9, 1980
32. JOHNSON RJ, IIDA H, ALPERS CE, MAJESKY MW, SCHWARTZ SM, PRITZL P, GORDON K, GOWN AM: Expression of smooth muscle cell phenotype by rat mesangial cells in immune complex nephritis. *J Clin Invest* 87:847–858, 1991
33. LEENAERTS PL, STAD RK, HALL BM, VAN DAMME BJ, VANRENTERGHEM Y, DAHA MR: Hereditary C6 deficiency in a strain of PVG/c rats. *Clin Exp Immunol* 97:478–484, 1994
34. BRAUER RB, BALDWIN WM, DAHA MR, PRUITT SK, SANFILIPPO F: Use of C6-deficient rats to evaluate the mechanism of hyperacute rejection of discordant cardiac xenografts. *J Immunol* 151:7240–7248, 1993
35. BRAUER RB, BALDWIN WM, IBRAHIM S, SANFILIPPO F: The contribution of terminal complement components to acute and hyperacute allograft rejection in the rat. *Transplant* 59:288–293, 1995
36. BAKER PJ, ADLER S, YANG Y, COUSER WG: Complement activation by heat-killed human kidney cells: Formation, activity and stabilization of cell-bound C3 convertases. *J Immunol* 133:877–880, 1984
37. QUIGG RJ, CYBULSKY AV, SALANT DJ: Effect of nephritogenic antibody on complement regulation in cultured rat glomerular epithelial cells. *J Immunol* 147:838–845, 1991
38. HANSCH GM, SCHIEREN G, WAGNER C, SCHONERMARK M: Immune damage to the mesangium: Antibody- and complement-mediated stimulation and destruction of mesangial cells. *J Am Soc Nephrol* 2(Suppl 10):S139–S143, 1992
39. WAGNER C, BRAUNGER M, BEER M, ROTHER K, HÄNSCH GM: Induction of matrix protein synthesis in human glomerular mesangial cells by the terminal complement complex. *Exp Nephrol* 2:51–56, 1994
40. TORBOHM I, SCHONERMARK M, WINGEN A-M, BERGER B, ROTHER K, HÄNSCH GM: C5b-8 and C5b-9 modulate the collagen release of human glomerular epithelial cells. *Kidney Int* 37:1098–1104, 1990
41. NUNEZ D, CHARRIAUT-MARLANGUE C, BAREL M, BENVENISTE J, FRADE R: Activation of human platelets through gp140, the C3d/EBV receptor (CR2). *Eur J Immunol* 17:515–520, 1987
42. COSGROVE LJ, D'APICE AJF, HADDAD A, PEDERSEN J, MCKENZIE IF: CR3 receptor on platelets and its role in the prostaglandin metabolic pathway. *Immunol Cell Biol* 65:453–460, 1987
43. VIK DP, FEARON DT: Cellular distribution of complement receptor type 4 (CR4): Expression on human platelets. *J Immunol* 138:254–258, 1987
44. MEUER S, ECKER U, HADDING U, BITTER-SUERMANN D: Platelet-serotonin release by C3a and C5a: Two independent pathways of activation. *J Immunol* 126:1506–1509, 1981
45. WIEDMER T, ESMON CT, SIMS PJ: Complement proteins C5b-9 stimulates procoagulant activity through platelet prothrombinase. *Blood* 68:875–880, 1986
46. HALPERIN JA, TARATUSKA A, NICHOLSON-WELLER A: Terminal complement complex C5b-9 stimulates mitogenesis in 3T3 cells. *J Clin Invest* 91:1974–1978, 1993
47. ARNAOUT MA: Cell adhesion molecules in inflammation and thrombosis: Status and prospects. *Am J Kidney Dis* 21:72–76, 1993
48. NISHIKAWA K, GUO Y-J, MIYASAKA M, TAMATANI T, COLLINS AB, SY MS, MCCLUSKEY RT, ANDRES G: Antibodies to intercellular adhesion molecule 1/lymphocyte function-associated antigen I prevent crescent formation in rat autoimmune glomerulonephritis. *J Exp Med* 177:667–677, 1993
49. COUSER WG, SCHULZE M, PRUCHNO CJ: Role of C5b-9 in experimental membranous nephropathy. *Nephrol Dial Transplant* 1:25–31, 1992
50. FALK RJ, DALMASSO AP, KIM Y, TSAI CH, SCHEINMAN JI, GEWURZ H, MICHAEL AF: Neoantigen of the polymerized ninth component of complement. Characterization of a monoclonal antibody and immunohistochemical localization in renal disease. *J Clin Invest* 72:560–573, 1983
51. RAUTERBERG EW, LIEBERKNECHT HM, WINGEN AM, RITZ E: Complement membrane attack (MAC) in idiopathic IgA glomerulonephritis. *Kidney Int* 31:820–829, 1987